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(54) Title: PROCESS FOR PREPARATION OF FUNCTIONALIZED FIBER

(57) Abstract: A method for functionalizing a nanoweb having mean flow pore size from 0.1 and 5 μm and a porosity from 40 to 90 volume % to produce a functionalized nanoweb is described. The method comprises steps of: a) exposing the nanoweb and compound comprising at least two maleimido groups to an atmospheric plasma to produce a coated nanoweb; and b) exposing the coated nanoweb to a molecule capable of forming covalent bonds.

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PROCESS FOR PREPARATION OF FUNCTIONALIZED FIBER

Background

This invention relates generally to a method for producing a functionalized nanoweb useful in chromatography.

5 During the production of biopharmaceuticals, in particular antibody derived therapeutics, e.g., monoclonal antibodies, an affinity capture and elute purification step is frequently employed. This step most typically involves the binding of the desired therapeutic by a protein ligand conjugated to a chromatography support. Some support materials require surface functionalization prior to attachment of the protein ligand to the functionalized support surface.

10 Fibers have been used as support materials, e.g., in Z. Ma et al., J. Chromatogr. B, 877 (2009) 3686-3694, fibers are exposed to an air plasma in the presence of methacrylic acid to produce a functionalized fiber. In a separate step, in a liquid-phase reaction, a protein is bound to the functionalized fiber via amino groups on the protein.

Statement of Invention

15 The present invention is directed to a method for functionalizing a nanoweb having mean flow pore size from 0.1 and 5 μm and a porosity from 40 to 90 volume % to produce a functionalized nanoweb comprising a protein; said method comprising steps of:

- 20 a) exposing said nanoweb and a compound comprising at least two maleimido groups to an atmospheric plasma to produce a coated nanoweb; and
- b) exposing the coated nanoweb to a molecule capable of forming covalent bonds, preferably a protein.

25 The present invention is further directed to a functionalized nanoweb comprising a polymer and a nanoweb having mean flow pore size from 0.1 and 5 μm and a porosity from 40 to 90 volume %; wherein the polymer comprises maleimido groups.

Detailed Description

All percentages are weight percentages (wt%), and all temperatures are in °C, unless otherwise indicated. Averages are arithmetic averages unless indicated otherwise. All operations are performed at room temperature (from 18 to 25 °C) unless specified otherwise. A

5 carbohydrate polymer is a polymer comprising polymerized units of sugar molecules, i.e., a polysaccharide; polysaccharides functionalized via ether or ester groups are considered to be within this definition. A “maleimido” group is the substituent group corresponding to maleimide with the N-H hydrogen removed through substitution by a carbon atom.

10 A web of randomly distributed fibers is commonly referred to as a “nonwoven.” The fibers can be bonded to each other or unbonded, preferably unbonded. A “nanoweb” is a nonwoven web comprising at least one nanofiber. A nanoweb may also be referred to as a “nanofiber mat.” Preferably, the nanofibers in the nanoweb are “continuous,” i.e., having been laid down in one continuous stream to form the web. Fiber diameters may be determined by SEM picture examination. Preferably the fiber has a diameter from 0.1 to 1 μm. Preferably, at
15 least 95% of the fiber has a diameter in the stated range. Preferably, if less than 95% of the fiber has a diameter in the stated range, then fiber diameter is the arithmetic average of at least 50 measurements, preferably at least 100 measurements.

Preferably, the nanoweb has a mean flow pore size of at least 0.15 μm, preferably at least 0.2 μm, preferably at least 0.25 μm; preferably no more than 2 μm, preferably no more than 1
20 μm, preferably no more than 0.5 μm, preferably no more than 0.4 μm. Mean flow pore size is a calculated quantity from material porometry measurements, where the dry sample is subjected to airflow at various flow rates, then wetted with a fluid of known surface tension and air flows are returned at steadily increasing flow rate until the last wetted pore of the material is evacuated with air. A mean flow pore is determined from a ½ slope of the dry air flow curve intersecting
25 the wet flow curve. Relationships between fiber diameter and mean flow pore size have been determined. Simmonds et al proposed the following relationship to determine mean flow pore size, $D_{\text{mean,pore}}$ from the mean fiber diameter of the nonwoven, D_{fiber} (see Simmonds GE, Bomberger JD, Bryner MA. Designing nonwovens to meet pore size specifications. J Eng Fibers Fabrics. 2007;2(1),1-15.)

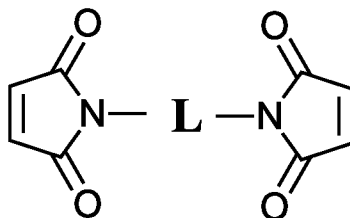
30 $D_{\text{mean,pore}} = 0.39267 * D_{\text{fiber}} / (1 - \text{porosity})$

Preferably, the nanoweb has a porosity from 40 to 90 volume %, preferably at least 50 volume%, preferably at least 60 volume %, preferably at least 65 volume%; preferably no more than 85 volume%, preferably no more than 80 volume%, preferably no more than 75 volume%. It is believed that fluid flow through the nanofiber mat is facilitated by high porosity, and binding capacity of a substrate is improved when the pore size is small. Preferably when pore size is 0.1 to 1 micron, porosity is 50 to 90 volume%; preferably when pore size is 0.1 to 0.5 micron, porosity is from 65 to 85 volume% Porosity is calculated from the following equation:

Porosity = $1 - (\text{mass of fiber in g/cm}^2 / (\text{Thickness of nanofiber mat in cm} * \text{Polymer Density in g/cm}^3))$

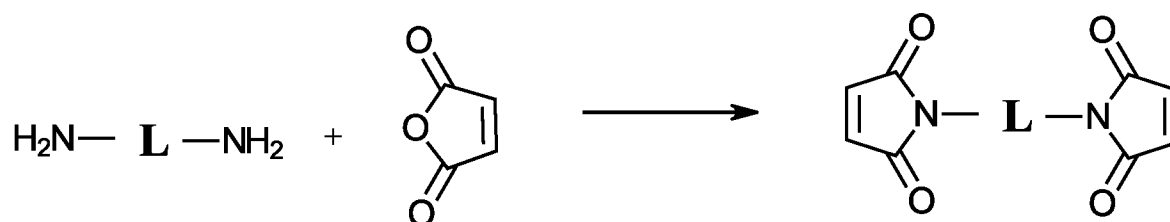
Preferably, the fiber comprises a synthetic polymer; preferably poly(vinylidene fluoride) (PVDF), copolymers of PVDF with vinyl monomers (preferably halogenated vinyl monomers, preferably poly(vinylidene fluoride-co-trifluoroethylene), polyamide, polyethersulfone (PES), polyethylene, polypropylene, polyester, polyimide or a combination thereof; preferably the fiber comprises PVDF, polyethersulfone, nylon or a combination thereof. Preferably, a PVDF polymer has a number average molecular weight is from 100,000 to 2,000,000 daltons, preferably from 200,000 to 500,000. Preferably, a polyamide has a number average molecular weight from 5,000 to 40,000, preferably from 10,000 to 20,000. Preferably, a polyethersulfone has a number average molecular weight from 20,000 to 80,000, preferably from 40,000 to 60,000. In a preferred embodiment of the invention the fiber comprises PVDF; preferably the fiber comprises at least 50 wt% PVDF, preferably at least 80 wt%, preferably at least 90 wt%, preferably at least 95 wt%.

Preferably, the compound comprising at least two maleimido groups has a molecular weight no greater than 1200 Daltons, preferably no greater than 900 Daltons, preferably no greater than 800 Daltons, preferably no greater than 600 Daltons. Preferably, the compound comprising at least two maleimido groups comprises two or three maleimido groups, preferably exactly two maleimido groups. Preferably, the compound has the structure:



wherein L is a difunctional organic substituent comprising from 2 to 20 carbon atoms. Such a structure would result from allowing a diamine or triamine to react with maleic acid (MA) or maleic anhydride (MANh), e.g.,

5



Preferably, L comprises no atoms other than carbon, hydrogen, oxygen and nitrogen atoms; preferably no atoms other than carbon, hydrogen and oxygen. Preferably, L has a molecular weight no greater than 1000 Daltons, preferably no greater than 700 Daltons, preferably no greater than 600 Daltons, preferably no greater than 500 Daltons. In a preferred embodiment of the invention, L is a difunctional hydrocarbyl group, i.e., a hydrocarbon molecule with two hydrogen atoms removed; preferably a linear aliphatic difunctional hydrocarbyl group, preferably with attachment points at the ends of the linear hydrocarbyl group, or an aromatic difunctional hydrocarbyl group having one or two aromatic rings.

Preferred examples of compounds comprising at least two maleimido groups include, but are not limited to: N,N'-hexamethylenediamine bismaleimide, N,N'-butanediaminebismaleimide, N,N'-ethylenediaminebismaleimide, 4,4'-bismaleimidodiphenylmethane, 2,4-bismaleimidotoluene, 1,4-bismaleimidobenzene, 1,3-bismaleimidobenzene, tris(2-maleimidoethyl)amine [139112-38-2] and combinations thereof.

The molecule capable of forming covalent bonds can include, but are not limited to, molecules that include nucleophilic groups. These nucleophilic groups can include, but are not limited to, hydroxyl, amine or thiol groups. These groups can be found in amino acids, peptides, proteins, nucleic acids, polynucleic acids, viruses and cells among others. Preferably the molecule capable of forming a covalent bond is a protein.

In one embodiment of the invention, the coated nanoweb is contacted with the protein in a plasma, preferably by forming an aerosol from an aqueous solution of protein and contacting the aerosol with the coated nanoweb in the presence of an atmospheric plasma. In a preferred

embodiment of the invention, the coated nanoweb is contacted with the protein in an aqueous medium. Preferably, the protein is capable of binding other proteins to an active site, and comprises a free thiol group. Preferably, the aqueous medium comprising the protein also comprises inorganic salts in a concentration from 1 to 2.25M, preferably from 1.25 to 2.25M.

- 5 Preferred salts are those recognized in the literature as having antichaotropic activity. Examples of antichaotropic salts include sodium sulfate, ammonium sulfate, and magnesium or zinc salts, including chloride, phosphate and carbonate.

The present invention is further directed to a functionalized nanoweb comprising a polymer and a nanoweb having mean flow pore size from 0.1 and 5 μm and a porosity from 40 to
10 90 volume %; wherein the polymer comprises maleimido groups. Preferences for mean flow pore size and porosity are the same as those stated previously in the description of the method. Preferably, the fiber comprises PVDF, preferably the fiber comprises at least 50 wt% PVDF, preferably at least 80 wt%, preferably at least 90 wt%, preferably at least 95 wt%. Preferably, the polymer forms a continuous coating covering at least 25% of the fiber surface, preferably at
15 least 50%, preferably at least 75%.

A thiol group in general, including a thiol group of a protein, is believed to react with a maleimido group via a Michael addition reaction to produce a covalent bond between the protein and the thiosuccinimide group resulting from the addition. The present invention is directed also to a nanoweb having thiosuccinimide groups and covalently bound protein.

- 20 Preferably, the protein comprises a free cysteine thiol group, i.e., a thiol group (-SH) which is not part of a disulfide linkage, and said protein is active with respect to binding other proteins to an active site. Preferably, the protein comprising a free cysteine thiol group is obtained by cleaving a disulfide bond in the protein which is not required for maintaining the integrity of the protein binding site, i.e., the protein's activity for binding other proteins.
- 25 Preferably, the disulfide is reduced using Tris(2-carboxyethylphosphine) hydrochloride (TCEP·HCl) which can be used at a pH between 1.5 and 9.0 depending upon the stability of the protein being reduced at that pH, preferably pH 3 to pH 8, using non-phosphate buffers e.g. TRIS, HEPES, Borate. Additional preferred reducing agents include dithiothreitol (DTT), mercaptoethanol (ME), 2-mercaptoethylamine hydrochloride (2-MEA.HCl) all of which are
30 typically utilized around neutral pH. These three reagents all include free thiol groups and can be removed from the protein by filtration prior to reacting the protein with the nanofibers to reduce competitive reactions during the conjugation of the protein, although use of TCEP·HCl is

preferred. Cysteine-Cysteine disulfide bonds are frequently found in proteins as a method of maintaining the protein structure such that cleavage of these disulfide bonds can result in denaturation of the protein. Other structural features such as β -sheets, helix bundles and hairpin structures can maintain a protein's conformation without the use of disulfide linkages.

- 5 Preferably, proteins useful for affinity binding of other proteins or useful biotherapeutic molecules are produced recombinantly or isolated from natural sources. Preferably, the protein is one which has been produced recombinantly so as to include a disulfide linkage that is not involved in the configurational stability of the resulting protein; preferred proteins of this type include Protein A, Protein A/G and Protein G. Other preferred proteins include antibodies,
10 monoclonal and polyclonal, the recognition fragments F(ab') and F(ab')₂ which can be produced recombinantly or following an enzymatic treatment to cleave the fragments from the intact antibody (Rosenstein et.al., Curr Protoc Mol Biol. 2020 Jun;131(1):e119.doi: 10.1002/cpmb.119). Preferably, disulfide bonds which are reduced in the F(ab')₂ are distal to the binding site of the protein and can be cleaved to form the individual fragments without loss of the
15 ability to bind to other proteins, most likely because disulfide linkages that help to maintain the protein configuration occur within a secondary structure such as a β -sheet which maintains the protein configuration and protects these disulfides from reduction. Preferably, the protein is in an aqueous solution at a concentration from 5 to 50 mM; preferably the pH of the solution is from 8 to 9.5, preferably from 8.5 to 9.3, preferably from 8.7 to 9.1 and the molecular weight of the
20 protein is no greater than 150,000 Daltons, preferably no greater than 100,000 Daltons and preferably no greater than 75,000 Daltons.

- Preferably, a dielectric barrier discharge atmospheric pressure plasma process is used to attach the multifunctional monomer injected as liquid aerosol to the nanofiber substrate. The dielectric barrier discharge plasma process preferably is a homogenous glow discharge process.
25 Homogeneous glow discharge plasma processes are known in the art to produce spatially uniform low temperature electrons from injected gases at atmospheric pressures. Ions collide with the injected monomers producing ionized species that may self-polymerize in aerosol prior to substrate deposition. Preferred gases suitable for plasma generation include carbon dioxide, nitrogen, argon, and/or helium. The flow, of gases in plasma form and the injected monomer
30 aerosol, passes through a nozzle with a defined cross-sectional area. Preferably, for treating nonwoven roll goods, the nozzle is rectangular in geometry with an interelectrode gap and a width larger than the width of the nonwoven. The inter-electrode gap is preferably 0.5-10 mm,

preferably, 0.8-2mm. The cross-sectional area of the nozzle used in the examples below is 5 cm² with a nozzle width of 40cm. Preferably, gas flow rates range from 2-150 slm/cm², where slm is in units of standard liters per minute with standard conditions of a gas volume are at temperature of 0°C and pressure of 1 atm (101kPa), preferably from 60 to 100 slm/cm². Gas flow rates

5 through the plasma head will depend on plasma head size and substrate width to be coated with monomer as well as on environmental temperature and pressure of the gas. Injected monomer flow rates depend on the amount of monomer to be attached per area of substrate. High injection rates of monomer may lead to self-polymerization prior to deposition on the substrate. Preferably, injection rates of aerosolized monomer range from 0.2 slm/cm² to 5 slm/cm².

10 Dilutions of monomers may be achieved by one or both means of liquid solution mixing, by dissolution or suspension of a solid monomer in water or an appropriate solvent, and by introduction of greater gas flow into the liquid monomer pure compound, suspension, or solvent mixture. Preferred dilution ratios (solvent or carrier to monomer) range from of 2:1 to 50:1, preferably from 3:1 to 20:1. Preferably, alternating current powered covered electrodes generate

15 the plasma through a narrow gap at atmospheric pressures from 0.9-1.1 atm (91-111 kPa). Preferably, plasma source voltage ranges from 1-100 kV with preferred range of 5-30 kV. Preferably supplied power for initial activation and reaction with monomer is in the range of 10 to 1500W/cm², preferably 100 to 1000W/cm², preferably 150 to 800 W/cm². Preferably supplied power in the presence of protein is in the range of 5 to 300 W/cm², preferably 10 to 200 W/cm²,

20 preferably 15 to 100 W/cm². Preferably, the distance between the substrate and the plasma head is 1-10 mm, preferably 2-5 mm. Preferably, the substrate is pretreated using carbon dioxide, nitrogen, argon, and/or helium in an atmospheric plasma process without monomer.

EXAMPLES

A nonwoven web was prepared using PVDF by a known electrospinning process. The resulting nanoweb had a porosity of 73% and mean flow pore size of 0.2 μm . The PVDF nanofiber nonwoven web is subjected to an activation pass through the plasma treatment device, in which the top and bottom surfaces of the web are exposed to a plasma of CO_2 and N_2 at a flow rate of 80 slm/ cm^2 and 8 slm/ cm^2 power level of 300 W/ m^2 . The activated PVDF web passes through a N_2 plasma at flow rate of 80 slm/ cm^2 and 200 W/ cm^2 with 4,4'-bismaleimidodiphenylmethane injected into the plasma as aerosol of 15 wt% solution with dimethyl sulfoxide. The monomer injection has a flow rate of 1 slm/ cm^2 and is diluted with N_2 gas at a ratio of 7:1. The distance between the electrode and substrate is about 2 mm. A measure of amount of monomer injected per mass of substrate is determined by gravimetric means of weighing the supply solution at the start and finish of the plasma process taking the difference between start and finish and dividing the difference by the mass of substrate processed

The maleimide coated samples were conjugated with a reactive cysteine on recombinant Protein A. This reaction involves first reducing disulfide bonds in the Protein A dimer using TCEP-HCl (Tris(2-carboxyethyl)phosphine hydrochloride) [See: Thermo Fisher Application Notes: <https://www.thermofisher.com>] in aqueous solution at 5 - 50 mM concentration and pH 6-8. The Protein A with a free reactive thiol is then allowed to react with the maleimide-activated nanofiber mat in the presence of a salting out buffer e.g. sodium sulfate to increase the coating of the Protein A on the surface of the fibers and as a result increasing the local concentration of Protein A at the surface of the fiber which increases the amount of Protein A conjugated onto the nanofiber mat [See Thermo Fisher Application Notes: <https://www.thermofisher.com>]

The saturated binding capacity, SBC, can be measured by placing a 2.5 cm diameter disc of the membrane into a Swinnex filter holder and using an AKTA PureTM, elute a 10 mL solution of human IgG (0.2 mg/mL) in PBS buffer, pH 7.4 at 0.5 ml/min through the membrane. The filter is then washed with about ten bed volumes of the PBS buffer and then the human IgG is eluted from the membrane with 5 bed volumes of buffer (e.g. 0.1 M glycine at pH 3), quickly neutralized to about pH 7 to avoid denaturation and the concentration measured spectrophotometrically, using the formula below or by integration of the elution curve and

comparing with a calibration curve (e.g. Hahn et. al. Journal of Chromatography A. 2005, 1093, 98):

Protein concentration

5

Protein concentration (mg/ml): Absorbance $\times$ dilution $\times$ (Molecular weight / Molar extinction coefficient)

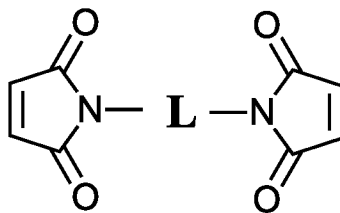
10

Example	Amount of agent injected / g substrate	SBC / membrane volume		Substrate Polymer
	mg/g	mg/cm ³		
Example 1	182	35.4		PVdF

15

Claims

1. A method for functionalizing a nanoweb having mean flow pore size from 0.1 and 5 μm and a porosity from 40 to 90 volume % to produce a functionalized nanoweb; said method comprising steps of:
 - 5 a) exposing said nanoweb and a compound comprising at least two maleimido groups to an atmospheric plasma to produce a coated nanoweb; and
 - b) exposing the coated nanoweb to a molecule capable of forming covalent bonds.
2. The method of claim 1 in which the nanoweb is not a carbohydrate polymer.
3. The method of claim 1 or claim 2 in which the nanoweb comprises poly(vinylidene fluoride) (PVDF), copolymers of PVDF with vinyl monomers, polyamide, polyethersulfone (PES), polyethylene, polypropylene, polyester, polyimide or a combination thereof.
4. The method of any preceding claim in which the compound comprising at least two maleimido groups has a molecular weight no greater than 1200 Daltons.
5. The method of any preceding claim in which the molecule capable of forming covalent bonds comprises molecules that include at least one of hydroxyl, amine or thiol groups.
6. The method of any preceding claim in which the compound comprising at least two maleimido groups has structure:



wherein L is a difunctional organic substituent comprising from 2 to 20 carbon atoms.

7. The method of any preceding claim in which said molecules comprise at least one protein
8. The method of claim 7 in which the protein is capable of binding other proteins to an active site
9. A functionalized nanoweb produced by the method of any preceding claim.
10. A functionalized nanoweb comprising a polymer and a nanoweb having mean flow pore size from 0.1 and 5 μm and a porosity from 40 to 90 volume %; wherein the polymer comprises maleimido groups.
11. A functionalized nanoweb comprising a nanoweb having mean flow pore size from 0.1 and 5 μm and a porosity from 40 to 90 volume %; wherein the nanoweb comprises thiosuccinimide groups and covalently bound protein.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER		
INV.	B01J20/32	B01J20/28
	D06M14/18	C23C4/00
	B01J20/286	B05D1/00
		D06M10/02
ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
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Minimum documentation searched (classification system followed by classification symbols)		
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
EPO-Internal, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2019/060516 A1 (MARTIN RUSSELL [US] ET AL) 28 February 2019 (2019-02-28) paragraph [0193] - paragraph [0206] examples 1,2,5,15 paragraph [0010] - paragraph [0011] figures 6A,13A paragraph [0191] -----	9-11
X	US 2019/134606 A1 (MURATA DAI [JP] ET AL) 9 May 2019 (2019-05-09) paragraph [0061] - paragraph [0062] claims 1,5-7 -----	9-11
X	US 2003/198968 A1 (MATSON ROBERT S [US]) 23 October 2003 (2003-10-23) claims 1,21-26 paragraph [0046] - paragraph [0056] ----- -/-	1-11
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
4 April 2023		13/04/2023
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Kaluza, Nicoleta

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International application No

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2005/106477 A2 (VITO [BE]; PAULUSSEN SABINE [BE] ET AL.) 10 November 2005 (2005-11-10) the whole document -----	1-11
A	WO 2004/113872 A2 (UNIV COLUMBIA [US]; LEVICKY RASTISLAV [US] ET AL.) 29 December 2004 (2004-12-29) the whole document -----	1-11

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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